



Induction of callus from leaves and stems of *Trigonella foenum-graecum* varieties

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SUMMARY

Trigonella foenum-graecum L. (fenugreek) plants are widely used in medicine. *In vitro* cultivation of this species has the potential for a number of biotechnological applications, such as steroid sapogenin and diosgenin production. The aim of this study was to generate fenugreek cell cultures and optimise chemical and physical factors regulating their growth *in vitro*. Leaf and stem-originated calli from "spring-summer" and "winter" fenugreek varieties were obtained and their growth kinetics were determined and analysed. The presence of 2,4-D, kinetin and IAA ("three hormone system") were found to be essential for maintaining a high rate of fenugreek callus induction. Stem explants generally provided higher callus induction rate than leaf explants. Biomass accumulation in tested fenugreek varieties depended on specific combination of 2,4-D, kinetin and IAA that increased with sucrose levels and decreased under illumination. As a result, we have developed protocols for initiation, subcultivation and long-term maintenance of fenugreek cultures, which have a great potential for industrial production of fenugreek-based drugs.

Keywords: fenugreek, *Trigonella foenum-graecum*, plant cultures *in vitro*, callus induction, callus growth, phytohormones, sucrose, light.

INTRODUCTION

Trigonella foenum-graecum L. (*T. foenum-graecum*) is a medicinal plant whose seed and leaf extracts demonstrate pronounced antidiabetic (Phadnis *et al.* 2011, Jefferson 1999),

anticarcinogenic (Shabbeer et al. 2009, Sur et al. 2001), antihypertensive (Balaraman et al. 2006), hepatoprotective (Kaviarasan et al. 2007), immunomodulating (Bilal et al. 2003) and other therapeutic effects. Fenugreek pharmacological activity is related to a number of alkaloids, phenolics, proteins, amino acids, mucilaginous fibres, vitamins and steroid sapogenins (Barnes et al. 2007, Basch et al. 2003).

Diosgenin is one the most important sapogenins from *T. foenum-graecum*, which is used as a substrate for approximately 60% of steroid, hormone and cortisone syntheses in pharmaceutical industry worldwide (Acharya et al. 2010, Randhir et al. 2004). Diosgenin has been found to be a strong antitumor agent (Jayadev et al. 2004, Shabbeer et al. 2009). It also showed estrogen-like action on mammary glands and can be involved in the control of cholesterol metabolism (De and De 2011). There is considerable commercial interest in the cultivation of fenugreek as a diosgenin-containing plant and in the increase of the sapogenin content in this plant to minimise the cost of diosgenin-based drug production. An alternative approach will be the development and optimisation of procedures for the cell cultures of *T. foenum-graecum*, which can provide industrialists with cheap and abundant fenugreek-derived material. Cell culture techniques are very convenient because they allow the production of commercially valuable substances *in vitro* (independently of agricultural seasons) and solve numerous problems arising from field cultivation, such as inadequate extraction and purification. Moreover, routine optimisation of cultivation conditions for cell culture can potentially increase the content of diosgenin in the plant material (Chawla 2002, Endreb 1994). This additionally decreases the costs of the production of this substance. The aim of this study was to initiate and optimise (by altering growth conditions and concentrations of growth regulators) the fenugreek calli in order to increase its growth rate. Leaf and stem-originated cultures from two fenugreek varieties have been obtained and their kinetics have been determined and analysed. As a result, we have developed new efficient protocols for initiation, subcultivation and long-term maintenance of *T. foenum-graecum* cultures, which have a great potential for industrial diosgenin production.

MATERIALS AND METHODS

T. foenum-graecum seeds of tax. conc. "winter" cultivar (c. v. variety): PSZ.G.SZ and tax. conc. "spring-summer" cultivar (c. variety): *Ovari 4*, were from our own collection (Professor Sándor Makai). "Winter" and "spring-summer" fenugreek varieties were aseptically cultivated over 4–5 weeks. Leaf and stem fragments were excised from these plants and cultivated in medium with phytohormones to initiate callus formation (Jha and Ghosh 2005, Chawla 2002). The sterile growth medium contained full strength Murashige and Skoog medium (MS) (Muragise and Skoog 1968), 3% sucrose and 0.08% microbiology grade agar (pH 5.7–5.8 adjusted with NaOH/HCl) (Mineo 1990, Jha and Ghosh 2005). Cultivation of the explants and calli were carried out in the dark at 24.5 °C.

Phytohormones auxins and cytokinins are essential for initiation and maintenance of calli growth (Endreb 1994, Misawa 1994). However optimal auxin and cytokinin concentrations

are different for different plant species. The optimal ratio of these hormones should be determined empirically (Bhojwani and Razdan 1996). Here, the effect of hormonal treatments on callusogenesis was tested using leaf explants of "spring-summer" *T. foenum-graecum* variety. Cytokinin kinetin and auxin 2,4-dichlorophenoxyacetic acid (2,4-D) were applied in four combinations: 1 mg l⁻¹ 2,4-D and kinetin, 1 mg l⁻¹ 2,4-D and 2 mg l⁻¹ kinetin, 2 mg l⁻¹ 2,4-D and 1 mg l⁻¹ kinetin, 2 mg l⁻¹ 2,4-D and kinetin.

To examine the efficacy of callus induction, the number of explants with callus formation per total number of explants planted was measured. Specific growth rate (SGR, g day⁻¹) and biomass doubling time (BDT, days) were determined using values of the fresh weight of callus measured on the first and last day of cultivation in the fresh medium (sub-cultivation period was 30–35 days) (Godoy-Hernández and Vázquez-Flota 2006, Mustafa et al. 2011). In all experiments, 3–4 independent replicates were tested and statistically analysed (Student's *t* test). P-values less than 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

The initial tests were carried out with "spring-summer leaf" variety. This allowed us to find out the initial growth conditions to start the optimisation process. Tests included an examination of the effect of phytohormones (different concentrations of 2,4-D and kinetin) and the addition of IAA (2 mg l⁻¹) to media (on top of 2,4-D and kinetin) (Table 1.). It has been found that all the concentrations of 2,4-D and kinetin stimulated the formation of calli (Figure 1.), with IAA inducing significant (up to 37±3%) increase in the probability of callus generation. Calli that have been cultivated in the presence of IAA ("three hormone system": 2,4-D/kinetin/IAA) demonstrated a soft friable texture in contrast to calli obtained in the presence of 2,4-D/kinetin-containing media (which had a very hard, dense structures).

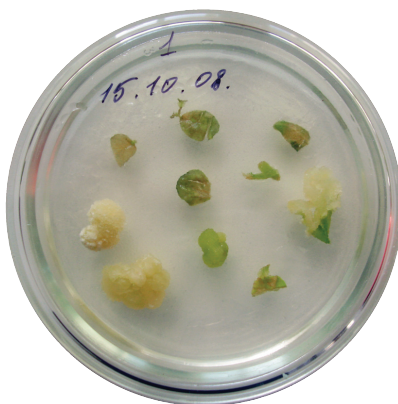


Figure 1. Callus formation from "spring-summer leaf" explants of *T. foenum-graecum*

Table 1. Effect of 2 mg l⁻¹ IAA on the efficacy of callus formation from "spring-summer leaf" explants of *T. foenum-graecum*

| 2 mg l ⁻¹ IAA | Efficacy of callus formation (%) | | | |
|--------------------------|----------------------------------|----------|----------|----------|
| | Variant of medium | | | |
| | 1 | 2 | 3 | 4 |
| – | 76.2±4.5 | 57.4±3.2 | 73.0±6.3 | 63.7±7.4 |
| + | 94.0±3.1 | 91.3±5.2 | 94.7±2.7 | 87.3±2.9 |

Values are means ± standard errors

1 – 1 mg l⁻¹ 2,4-D + 1 mg l⁻¹ kinetin; 2 – 2 mg l⁻¹ 2,4-D + 1 mg l⁻¹ kinetin;

3 – 1 mg l⁻¹ 2,4-D + 2 mg l⁻¹ kinetin; 4 – 2 mg l⁻¹ 2,4-D + 2 mg l⁻¹ kinetin

The observed "softening" of callus can be very important for the potential initiation of suspension cultures, which can only be obtained from calli with friable (loose) textures (Chawla 2002). Thus, this result of our study potentially has a significant biotechnological output (it can be used for developing techniques for the generation of suspension cultures). The optimal conditions ("three hormone system"), that were obtained for the "spring-summer leaf" variety have been applied to other explants ("spring-summer stem", "winter leaf" and "winter stem", respectively). Successful callus generation has also been found for these explants. All the hormone combinations induced calli (Table 2.). Comparison of the growth of different calli (four types: "spring-summer leaf", "spring-summer stem", "winter leaf" and "winter stem") showed that the probability of callus generation is significantly higher in the case of stem calli (98–100%). Stem calli showed a light yellow colour and "loose" texture, while calli derived from leaf explants demonstrated a brown colour and more dense texture.

Table 2. Effect of different combinations of 2,4-D and kinetin in the medium on the efficacy of *T. foenum-graecum* callus formation (%)

| Variant of medium | Efficacy of callus formation (%) | | | |
|-------------------|----------------------------------|---------------|----------------------|----------------------|
| | "winter leaf" | "winter stem" | "spring-summer leaf" | "spring-summer stem" |
| 1 | 90.3±5.8 | 98.3±1.8 | 94.0±3.1 | 97.7±2.3 |
| 2 | 94.7±2.7 | 100.0±0.0 | 91.3±5.2 | 97.7±3.3 |
| 3 | 88.3±2.7 | 97.7±2.3 | 94.7±2.7 | 100.0±0.0 |
| 4 | 91.0±2.0 | 100.0±0.0 | 87.3±2.9 | 98.0±2.0 |

Values are means ± standard errors

1 – 1 mg l⁻¹ 2,4-D + 1 mg l⁻¹ kinetin; 2 – 2 mg l⁻¹ 2,4-D + 1 mg l⁻¹ kinetin;

3 – 1 mg l⁻¹ 2,4-D + 2 mg l⁻¹ kinetin; 4 – 2 mg l⁻¹ 2,4-D + 2 mg l⁻¹ kinetin

Although the initial induction of calli showed a similar probability at different hormone combinations (in the "three hormone system" and with all explants), the callus growth and biomass accumulation (measured after induction) varied in calli from different origins and different hormone mixtures. Calli of "spring-summer leaf", "spring-summer stem", "winter leaf" and "winter stem" varieties demonstrated the most pronounced biomass increase in media containing 2 mg l⁻¹ 2,4-D and kinetin, 1 mg l⁻¹ 2,4-D and kinetin, 1 mg l⁻¹ 2,4-D and 2 mg l⁻¹ kinetin, 2 mg l⁻¹ 2,4-D and kinetin, respectively (Table 3.).

Table 3. Effect of different combinations of 2,4-D and kinetin in the medium on the growth of *T. foenum-graecum* calli

| Variant of medium | "winter leaf" | | "winter stem" | | "spring-summer leaf" | | "spring-summer stem" | |
|-------------------|----------------------------|-------------|----------------------------|-------------|----------------------------|-------------|----------------------------|-------------|
| | SGR g day ⁻¹ | BDT days | SGR g day ⁻¹ | BDT days | SGR g day ⁻¹ | BDT days | SGR g day ⁻¹ | BDT days |
| 1 | 0.039±0.003 | 18.61±1.32 | 0.025±0.002 | 27.74±1.51 | 0.043±0.005 | 18.92±2.47 | 0.045±0.003 | 15.90±1.21 |
| 2 | 0.029±0.003 | 24.84±2.51 | 0.029±0.002 | 24.24±1.64 | 0.043±0.009 | 22.81±2.98 | 0.018±0.001 | 39.64±2.54 |
| 3 | 0.066±0.005 | 11.21±0.84 | 0.037±0.004 | 21.11±2.30 | 0.055±0.006 | 14.08±1.30 | 0.014±0.002 | 52.37±6.26 |
| 4 | 0.032±0.002 | 21.93±1.24 | 0.060±0.007 | 13.04±0.94 | 0.062±0.007 | 11.69±1.16 | 0.022±0.004 | 36.80±6.67 |

Values are means ± standard errors

1 – 1 mg l⁻¹ 2,4-D + 1 mg l⁻¹ kinetin; 2 – 2 mg l⁻¹ 2,4-D + 1 mg l⁻¹ kinetin;

3 – 1 mg l⁻¹ 2,4-D + 2 mg l⁻¹ kinetin; 4 – 2 mg l⁻¹ 2,4-D + 2 mg l⁻¹ kinetin

The minimal and maximal biomass doubling time was found for "winter leaf" (11.21 days) and "spring-summer stem" (15.9 days).

The next stage of our work was to examine the effect of carbohydrates on the growth rate of calli ("carbohydrate optimisation stage"). Sucrose is a key component of most commercial growth media that are used for the cultivation of isolated plant cells and tissues (Chawla 2002, Bhojwani and Razdan 1996). Here, the effects of several sucrose concentrations (2, 3, 4 and 5% sucrose) were examined in media that were preliminary optimized for hormone combination (see above; Table 3). 4% sucrose was found to be optimal for cultivation in all calli (Table 4). In some cases, the addition of 5% sucrose resulted in a similar or lower rate of biomass increase that was probably related to osmotic stress caused by this sucrose level (Huang and Liu 2002, Farrukh and Sumaira 2008). Overall, media supplied with 4 and 5% sucrose promoted 1.5–2.4-fold increase in growth rate of calli as compared to 2 and 3% sucrose-supplemented media. According to published data, the stimulatory effect of sucrose on plant cell culture growth is associated with the increased availability of "highly energetic" organic substrate that can be directly used for anabolic reactions (Mathes et al. 1973). Sucrose has also been shown to increase the duration of the stationary phase of growth cycle, auxin production and enzyme activities of the pentose phosphate pathway (Endreb 1994).

Table 4. Effect of sucrose content in the medium on the growth of *T. foenum-graecum* calli

| Sucrose content (%) | "winter leaf" | | "winter stem" | | "spring-summer leaf" | | "spring-summer stem" | |
|---------------------|----------------------------|-------------|----------------------------|-------------|----------------------------|-------------|----------------------------|-------------|
| | SGR g day ⁻¹ | BDT days | SGR g day ⁻¹ | BDT days | SGR g day ⁻¹ | BDT days | SGR g day ⁻¹ | BDT days |
| 2 | 0.061±0.007 | 12.03±0.64 | 0.053±0.007 | 14.21±0.85 | 0.063±0.008 | 12.02±1.34 | 0.032±0.005 | 22.89±1.35 |
| 3 | 0.066±0.005 | 11.21±0.84 | 0.060±0.007 | 13.04±0.94 | 0.062±0.007 | 11.69±1.16 | 0.045±0.003 | 15.90±1.21 |
| 4 | 0.141±0.022 | 5.11±0.73 | 0.144±0.013 | 4.96±0.46 | 0.146±0.027 | 5.05±0.87 | 0.070±0.005 | 9.96±0.66 |
| 5 | 0.098±0.009 | 7.08±0.68 | 0.085±0.006 | 8.17±0.61 | 0.051±0.006 | 13.83±1.67 | 0.070±0.006 | 9.85±0.75 |

Values are means ± standard errors

Transferring calli to light, can have stimulatory or inhibitory effects on growth (Anasori and Asghari 2008, George et al. 2008, Lavee and Messer 1969). Growth of stem callus of the "spring-summer" fenugreek variety does not show significant changes after transferring

to light (14 h light/10 h dark; 3000 lux), while the growth of "spring-summer leaf", "winter leaf" and "winter stem" calli decreased by 57%, 50% and 58%, respectively (Table 5). The decrease of the growth rate under illumination could be related to light-induced changes in plant cell metabolism. It can be explained by the fact that light affects metabolic processes in plant cells, such as the formation of plastid ultrastructure, synthesis of chlorophyll, plastid membrane constituents and Calvin cycle enzymes (Kasemir 1979). Another mechanism is the activation of the phytochrome signalling pathway, which can modify the mitotic activity and cell development in calli (Davidson and Yeoman 1974). Therefore cultivation media for calli with light-induced changes in metabolic state, might require an "optimisation step" for cultures growing in the presence of light.

Table 5. Effect of light on the growth of *T. foenum-graecum* calli

| Light conditions | "winter leaf" | | "winter stem" | | "spring-summer leaf" | | "spring-summer stem" | |
|------------------|----------------------------|-------------|----------------------------|-------------|----------------------------|-------------|----------------------------|-------------|
| | SGR g day ⁻¹ | BDT days | SGR g day ⁻¹ | BDT days | SGR g day ⁻¹ | BDT days | SGR g day ⁻¹ | BDT days |
| dark | 0.141±0.022 | 5.11±0.73 | 0.144±0.013 | 4.96±0.46 | 0.146±0.027 | 5.05±0.87 | 0.070±0.005 | 9.96±0.66 |
| light | 0.070±0.007 | 10.66±0.88 | 0.061±0.005 | 12.35±1.13 | 0.063±0.007 | 11.84±1.13 | 0.075±0.017 | 9.74±0.53 |

Values are means ± standard errors

The following conclusions can be drawn from the present study:

1. The presence of 2,4-D, kinetin and IAA ("three hormone system") is required for the maximal rate of *T. foenum-graecum* callus induction.
2. Stem explants provided a higher callus induction rate than leaf explants.
3. Biomass accumulation in both stem and leaf-derived calli from "spring-summer" and "winter" varieties depended on the specific combination of 2,4-D, kinetin and IAA (optimal combination has been found for each variety), increased in media with higher sucrose levels (4–5%) and decreased under illumination.

A különböző görögszénafajták (*Trigonella foenum-graecum* L.) levelének és szárának sejtindukciós vizsgálata

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ÖSSZEFOGLALÁS

A görögszéna (*Trigonella foenum-graecum* L.) a gyógyászatban széles körben használt gyógynövény. A növény *in vitro* tenyésztésének vizsgálata számos biotechnológiai alkalmazásra ad lehetőséget, úgy mind szteroid sapogenin-, diosgeninyártás. A tanulmány célja görögszéna sejt kultúrák előállítás, valamint kémiai és fizikai jellemzők optimalizálása a kémcsövekben való növekedés céljából. Levelekből, szárból származó sejtek „tavaszi” valamint „téli” fajtákból származnak és ezek növekedési kinetikája került meghatározásra és elemzésre. A 2,4-D és kinetin IAA („három hormonrendszer”) jelenlétét állapították meg, amely nélkülözhetetlen a görögszéna kalluszindukció magas arányának fenntartásához. A szárkivonatok általában magasabb kalluszindukció arányt biztosítottak, mint a levélkivonatok. A tesztelt fajtákban a biomassza-felhalmozódás 2,4-D, kinetin és IAA különböző kombinációi függvényében történt, szacharózszint növekedése, valamint megvilágítás csökkenése mellett. Ennek eredményeként, az általunk kifejlesztett módszerek alkalmazásuk másodlagos tenyésztés létrehozására és görögszénakultúrák hosszú távú fenntartására, amelyek nagy lehetőségeket rejtenek a görögszéna-alapú gyógyszerek ipari termelésében. **Kulcsszavak:** görögszéna, *Trigonella foenum-graecum*, *in vitro* növényi kultúrák, kalluszindukció, kallusznövekedés, fitohormonok, szacharóz, fény.

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